Bile salt homeostasis in normal and Bsep gene knock out rats with single and repeated doses of troglitazone

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Abbreviations
ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate transaminase; AUC: area under the curve; Baat: Bile acid-CoA:amino acid N-acyltransferase; Bcrp: breast cancer resistance protein; Bsep: bile salt export pump; BUN: blood urea nitrogen; CA: cholic acid; CDCA: chenodeoxycholic acid; Cmax: maximum plasma concentration; DCA: deoxycholic acid; DILI: drug-induced liver injury; Fxr: Farnesoid X receptor; GCA: glycocholic acid; GCDCA: glycochenodeoxycholic acid; GDCA: glycodeloxycholic acid; HPLC: high-performance liquid chromatography; KO: knock out; MCA: muricholic acid; Mrp: multidrug resistance-associated protein; Ntcp: Sodium taurocholate cotransporting polypeptide; Oatp: organic anion-transporting polypeptide; PAPS: adenosine 3’-phosphate-5’-phosphosulfate; P-gp: p glycoprotein; PPAR: peroxisome proliferator-activated receptor; PPIA: peptidylprolyl isomerase A; Shp: small heterodimer partner; t1/2: elimination half-life; TBIL: total bilirubin; TCA: taurocholic acid; TCDCA: taurochenodeoxycholic acid; TDCA: taurodeoxycholic acid; TGZ: troglitazone; TMCA: tauro-muricholic acid; Tmax: time reaching Cmax; TS: troglitazone sulfate; WT: wild type
Abstract

The interference of bile acid secretion through bile salt export pump (BSEP) inhibition was one of the mechanisms for troglitazone (TGZ) induced hepatotoxicity. Here we investigated the impact of single or repeated oral doses of TGZ (200 mg/kg/day, 7 days) on bile acid homoeostasis in wild-type (WT) and Bsep KO rats. Following oral doses, plasma exposures of TGZ were not different between WT and KO rats, and were similar on Day-1 and Day-7. However, plasma exposures of the major metabolite, troglitazone sulfate (TS), in KO rats were 7.6- and 9.3-fold lower than in WT on Day-1 and Day-7, respectively, due to increased TS biliary excretion. With Bsep KO, the mRNA levels of Mrp2, Mrp3, Mrp4, Mdr1, Bcrp, Ntcp, Shp, and Sult2A1 were significantly altered in KO rats. Following 7 TGZ daily treatments, Cyp7A1 was significantly increased in both WT and KO rats. In the vehicle groups, plasma exposures of individual bile acids demonstrated variable changes in KO rats as compared to WT. When dosed with TGZ, many bile acid species were increased in the plasma of WT rats on Day-1, suggesting the inhibition of Bsep. Conversely, these changes returned to base levels on Day-7. In KO rats, alterations of most bile acids were observed after 7 doses of TGZ. Collectively, bile acid homeostasis in rats was regulated through bile acid synthesis and transport in response to Bsep deficiency and TGZ inhibition. Additionally, we demonstrate, for the first time, that repeated TGZ doses can upregulate Cyp7A1 in rats.
Visual Abstract
Introduction

Drug induced liver injury (DILI) is one of most frequently observed adverse effects in clinical practices and can lead to acute liver failure and liver transplant (Leise et al., 2014). Meanwhile, many drugs have been withdrawn from the market due to severe DILI, such as iproniazid, ticrynafen, benoxaprofen, bromfenac, troglitazone (TGZ) and ximelagalan (Lammert et al., 2008). Causes of DILI are complicated and could be contributed by a single factor or multiple combinations, including drug physicochemical properties, pharmacokinetics, reactive metabolites, oxidative stresses, mitochondrial liabilities, immune responses and drug endogenous molecule interactions (Hussaini and Farrington, 2007).

Transporters are a class of membrane proteins which are critical for normal cell functions by regulating the intake of nutrients and physiological chemicals and the elimination of waste and toxins. Alteration of transporter activities can cause severe organ injuries or systemic toxicities in humans (Cheng et al., 2016a). For example, bile salt export pump (BSEP) is an adenosine triphosphate (ATP)-dependent transporter predominately expressed on the canalicular membrane of hepatocytes, where its major function is to mediate the efflux of bile acids from hepatocytes to the bile duct to maintain the normal bile acid homeostasis in the body (Meier et al., 1984; Stieger et al., 1992; Gerloff et al., 1998). Impaired BSEP function can lead to intrahepatic cholestasis in humans, as a result of intracellular accumulation of bile acids in hepatocytes. Several BSEP gene mutations are confirmed to be associated with progressive familial intrahepatic cholestasis type 2 (PFIC2) in humans, a progressive cholestasis usually necessitating liver transplantation (Jansen et al., 1999; Lang et al., 2007; Ho et al., 2010). On the other hand, inhibition of BSEP activity by drugs can result in similar consequences. For example, TGZ induced hepatotoxicity observed in the clinic is related to, at least partially, the inhibition of BSEP by TGZ and its sulfate conjugated...
metabolite (Izumi et al., 1996; Funk et al., 2001a; Funk et al., 2001c; Smith, 2003). Systemic investigations of hundreds of drugs have demonstrated the correlation between BSEP inhibition and DILI (Morgan et al., 2010; Morgan et al., 2013; Pedersen et al., 2013).

As drugs and/or metabolites with the capability to inhibit BSEP have the potential to cause DILI, a variety of in vitro assays have been developed in early drug discovery to screen BSEP inhibition, an effort to reduce the risk of hepatotoxicity in patients (Cheng et al., 2016b). However, translating in vitro BSEP inhibition data directly to human DILI outcomes remains challenging. Mouse and rat have gene sequences of Bsep conservative to human orthologs, but they are more tolerable than humans in the aspect of BSEP impairment related hepatotoxicity (Noe et al., 2001). For example, while humans with BSEP polymorphisms can develop PFIC2, Bsep knock out (KO) mice only display mild non-progressive intrahepatic cholestasis (Wang et al., 2001). Drugs like bosentan and TGZ are known to cause DILI in humans, but are well-tolerated in rodents at much higher plasma exposures. One possible reason is that the bile acid pool in rodents is more hydrophilic and less toxic (Perwaiz et al., 2003). Additionally, it has been reported that compensatory pathways in rodents are available to maintain bile acid homeostasis when there is a lack of Bsep activity (Wang et al., 2009). As such, characterizing the mechanisms associated with bile acid homeostasis can help understand the rodent capability to handle drugs that can cause DILI in humans.

Previously, we reported a Bsep KO rat model in which Bsep was knocked out by zinc finger nuclease technology in Sprague-Dawley rats (Cheng et al., 2016b). This Bsep KO rat model showed a normal appearance and regular activity. The bile flow was within the normal range of reference values. However, bile acids profiles were altered in this Bsep KO rat model and the liver has a reduced capacity to excrete administered taurocholic acid (TCA). In the present study, we investigated single or repeated oral doses of TGZ on bile salt homeostasis in rats. Following the
administration of TGZ at a dose of 200 mg/kg/day for up to 7 days, individual bile acid profiles were characterized in WT and Bsep KO rats. Additionally, gene expressions of liver transporters, enzymes and nuclear factors that are associated with bile acid disposition were also evaluated to compare with the bile acid profiles.
Methods

Chemicals and materials

Troglitazone (TGZ) was synthesized by Bristol-Myers Squibb (New Brunswick, NJ). Troglitazone Sulfate (TS) and pioglitazone was purchased from Sigma-Aldrich Co. (St. Louis, MO). Chenodeoxycholic acid (CDCA), cholic acid (CA), deoxycholic acid (DCA), glycocholic acid (GCA), glycodyeoxcholic acid (GDCA), glycochenodeoxycholic acid (GCDCDA), taurocholic acid (TCA), taurodeoxycholic acid (TCDA), and taurochenodeoxycholic acid (TCDCA) were purchased from Sigma-Aldrich (St. Louis, MO). α-muricholic acid (αMCA), tauro-α-muricholic acid (TαMCA), β-muricholic acid (βMCA) and tauro-β-muricholic acid (TβMCA) were purchased from Steraloid inc (Newport, RI). Deuterium labeled deoxycholic acid (D4-DCA) was purchased from CDN Isotopes (Quebec, Canada). Adenosine 3’-phosphate-5’-phosphosulfate (PAPS) and alamethicin were obtained from Sigma-Aldrich Co. (St. Louis, MO).

Study design

Wild type (WT) male Sprague-Dawley (SD) rats and male Bsep knockout (KO) SD rats were obtained from SAGE Labs (St. Louis, MO). All animals were maintained in the animal facility (Hopewell, NJ) with controlled temperature (25 °C), humidity (40-60%) and light/dark cycle (12h). The animal care and study protocols were approved by the Animal Care and Use Committee at Bristol-Myers Squibb (Hopewell, NJ). Rats were randomly assigned to 4 groups (n=5-6 each group): TGZ (200 mg/kg, 2 mL/kg) in KO rats, TGZ (200 mg/kg, 2 mL/kg) in WT rats, vehicle (2 mL/kg) in KO rats, and vehicle (2 mL/kg) in WT rats. TGZ was prepared as suspension in the dosing vehicle of 0.15% docusate sodium/PVP-K30/H2O (0.15%/2%/97.85%) at the concentration of 100 mg/mL. The particle size of 90% TGZ was less than 8 µm. The purity of
TGZ was greater than 99% and the formulation was stable for a week as checked by high-performance liquid chromatography (HPLC).

After at least a week of acclimation, the rat jugular vein was cannulated for blood collection. On the following day, rats received once daily oral dose through oral gavage for 7 days. Plasma samples were collected at 0, 0.5, 1, 2, 4, 7 and 24 h after the 1st and 7th dose. Serum was collected at 0 h (pre-dose) on Day-1 and 24h on Day-7. Withdrawn blood (~300 µL) was replaced with an equal volume of saline solution. At 24 h post the 7th dose, animals were euthanized by CO₂ inhalation and liver was collected from each animal. Plasma and liver samples were stored at -80 °C until analysis.

**TGZ and TS quantitation**

All samples were thawed from -80 °C freezers, and then processed and analyzed immediately to minimize the degradation of the test articles. Plasma samples (30 µL) were extracted with 60 µL of acetonitrile containing pioglitazone as internal standard. Liver samples were homogenized with 10x volume of blank rat plasma and then processed the same as plasma samples. Reference standards of TGZ and TS were spiked in control rat plasma and then processed the same as testing samples. The mixture was then vortexed and filtered through filter plates. With the addition of 60 µL of H₂O, an aliquot of the samples (5 µL) were injected onto a Shimadzu LC-10AD HPLC system (Shimadzu Scientific Instruments, Columbia, MD), connected with a Atlantis C18 column (5 μm, 2.1x50 mm). The initial mobile phase of 5% solvent B was changed to 20% solvent B over 1.5 min, then to 95% solvent B over 1 min with linear gradients; solvent A is 0.1% formic acid in water and solvent B is acetonitrile. The flow rate was 0.3 ml/min. The HPLC eluate was introduced into a Sciex Q-Trap 4000 mass spectrometer equipped with an electrospray ionization source operating at negative-ion mode. The specific transitions for monitored analytes were TGZ (m/z...
440.1→397.2), TS (m/z 520.1→440.4) and pioglitazone (m/z 355→311). In the evaluation of this analytical method, no carryover was observed between two sample injections. The lower limits of quantitation were found to be 30 nM and 10 nM for TGZ and TS, respectively. The ion responses were reliable up to 10 µM within 20% of variance for both TGZ and TS. Concentrations of TGZ and TS were determined based on their respective standard curves and samples with a concentration greater than the highest standard concentration were diluted and reanalysed.

**TS formation in liver S9 incubation**

To compare the enzymatic activities towards formation of TS, the relative amount of TS was quantified following incubation with TGZ and liver S9 fraction from WT and KO rats treated with vehicle. Liver S9 was prepared by centrifuging the liver tissue (homogenized with 5x volume of phosphate buffer, pH 7.4) at 10000g for 30 min at 4°C. The protein concentration was measured using BCA protein assay (Thermo Scientific, Rockford, IL). The in vitro reaction was carried out in Tris-Cl buffer (pH 7.4, 100 mM) containing PAPS (~0.1 mM), alamethicin (25 mg/ml), and TGZ (10 µM). The mixture was pre-incubated for 5 minutes at 37°C and then initiated by adding S9 fraction (1 mg/mL). An aliquot of samples (100 µL) were collected at 5, 15, 30 and 60 min, and extracted with 200 µL of acetonitrile containing tolbutamide (100 nM), followed with filtration through filter plates. The supernatant was brought to near-dryness (not totally dry) under N2 and then reconstituted in 200 µL of H2O with 0.1% FA. Samples were analyzed using LC-MS/MS as described above. The specific MRM transition for tolbutamide was 269.1→169.8. The relative amount of TS was quantified based on the peak area ratio between TS and tolbutamide.

**Liver RNA isolation and RT-PCR analysis**
The gene expression of selected liver transporters, metabolic enzymes and nuclear receptors (as listed in Table 1) was measured in liver samples collected from Bsep WT and KO rats dosed with vehicle or TGZ for 7 days. For mRNA isolation, frozen liver was first homogenized in Trizol using 5 mm diameter stainless steel beads with TissueLyser (Qiagen, Valencia, CA). Total RNA was purified using the RNeasy® Mini Kit (Qiagen, Valencia, CA) including the on-column DNase treatment following the manufacturer’s protocol. The quality and quantity of liver total RNA were evaluated with a NanoDrop 8000 (Thermo Scientific, Wilmington, DE). cDNA was synthesized from 2 µg of total RNA using SuperScript® III Reverse Transcriptase (Life Technologies, Grand Island, NY).

Real-time PCR was performed in duplicate on the 384-well block format ViiA 7 Real-Time PCR System using Power SYBR® Green PCR Master Mix (Life Technologies, Grand Island, NY). PCR was performed with the default thermal profile using diluted cDNA equivalent to 16 ng of total RNA as the PCR template. The assayed genes with full names, GeneBank accession numbers, and PCR primer sequences are provided in Table 1. The mRNA expression of each gene was normalized to the housekeeping gene peptidylprolyl isomerase A (PPIA).

To confirm the Bsep knockout, two Taqman probes at the ZFN targeted mutation site were designed, one for the intact cDNA sequence and the other for the 8 nucleotide deleted sequence. PCR was performed as described above, except using Taqman Gene Expression Master Mix (Life Technologies, Grand Island, NY).

**Full-length Bsep cDNA sequence analysis**

To further verify that the ZFN targeted 8 nucleotide mutation region is intact in WT rats and is deleted in Bsep KO rats, full-length rat Bsep cDNA from all rats was sequenced. PCR
amplification was carried out using Platinum Taq High Fidelity DNA polymerase with the following thermo-cycling profile: 94°C for 2 minutes, followed by 35 cycles of 94°C for 15 seconds, 59°C for 30 seconds, and 68°C for 1 minute, and a final 68°C for 7 minutes. PCR amplicon products were analyzed by gel electrophoresis and the expected size bands were identified. The PCR products from each rat liver were sequenced to confirm sequence identity.

**Serum chemistry**

Serum chemistry tests were conducted for samples collected predose and 24 h post last dose. The samples were analyzed using the Siemens Advia 1800 automated chemistry instrument, and the tests included: aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin (TBIL), blood urea nitrogen (BUN), creatinine, cholesterol, triglycerides and albumin.

**Bile acids quantitation**

The concentrations of bile acids were determined in all plasma samples collected on Day-1 and Day-7, and in liver samples at the end of experiments. The monitored bile acids included CA, DCA, CDCA, GCA, GDCA, GCDCA, TCA, TDCA, TCDCA, αMCA, βMCA, TαMCA and TβMCA. The analytical methods were the same as reported previously (Cheng et al., 2016b).

**Data analysis**

The pharmacokinetic parameters of TGZ and TS in rat plasma were derived from plasma concentrations versus time data with a non-compartment model using Phoenix WinNonlin 6.3 (Certara, Princeton, NJ). The plasma exposure of individual bile acids was determined as the area under the plasma concentration–time curve from time zero to 24 h (AUC0-24) using non-compartmental methods. All data here are reported as mean ± SD, or as otherwise noted. Statistical
test was conducted using one-way ANOVA with Fisher’s LSD post analysis using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). Differences were considered significant when $p < 0.05$. 
**Results**

Following the first oral dose, TGZ was rapidly absorbed and the time of maximum plasma concentration (Tmax) was 1.8 h and 1.5 h in WT and KO rats, respectively (Figure 1A). The peak plasma concentration (Cmax), AUC_{0-24} and the elimination half-life (t_{1/2}) of single dose TGZ were comparable between WT and KO rats (Table 2). Repeated TGZ doses had minimal effects on the pharmacokinetics of TGZ as none of the parameters were significantly different between Day-1 to Day-7 in either WT rats or KO rats. In contrast, the Cmax and AUC_{0-24} of TS in plasma were significantly decreased in KO rats, about 7.6 and 17.9 fold lower than in WT rats on Day-1 (Figure 1B). Similar decreases of TS in the plasma were also observed on Day-7 in KO rats.

At the end of experiments (24 h post the last dose), liver samples were collected and the concentrations of TGZ and TS in the liver were measured. Although the liver concentrations of TGZ in both WT and KO rats were below the lower limit of quantitation (LLOQ, 30 nM) (Figure 2A), a substantial amount of TS was still detectable. Similar to the patterns observed in plasma, the liver concentrations of TS in KO rats were significantly lower than in WT rats. To determine whether the formation of TS is impacted by Bsep KO in rat, TGZ was incubated with liver S9 fractions from WT and KO rats. As shown in Figure 2B, the formation rate of TS in liver fraction were not different between WT and KO following the incubation at 5, 15, 30 and 60 min.

To confirm the genetic modification, Bsep mRNA expression was determined using RT-PCR in rat liver samples. When applying the probes targeting the ZFN modification region, the Bsep mRNA expression was only detectable in WT rats but not in KO animals (Figure 3A; Supplemental Figure 1A). On the other hand, the modified mRNA with the deletion of 8 nucleotides was only detected in KO rats (data not shown). Interestingly, a higher amount of Bsep mRNA expression
was observed in KO rats than in WT rats when the PCR was designed for the downstream of the ZFN target mutation region (Supplemental Figure 1B). In both WT and KO rats, TGZ has no effect on Bsep expression (Supplemental Figure 1A). Furthermore, the cDNA of Bsep from both WT and KO rats were sequenced and it was confirmed that the 8 nucleotides were removed in all studied KO rats. During the sequencing of rat full-length Bsep cDNA, a splice variant isoform of Bsep was identified in both WT and KO rats, which was a 49 basepair fragment spliced at 3' of exon 3 comparing to the RefSeq NM_031760 (Supplemental Figure 2).

The gene expression of major liver transporters (Mrp2, Mrp3, Mrp4, Bcrp, Mdr1, Ntcp, Oatp1a1) and enzymes/nuclear receptors (Fxr, Shp, Baat, Cyp7A1, Sult2a1) were also characterized (Figure 3). In the rats administered with vehicle, the mRNA levels of Mrp2, Mrp3, Mdr1, Baat, Ntcp, Shp and Sult2A1 in KO rats were significantly increased, while Bcrp was decreased when compared with WT rats. In both WT and KO rats, repeated doses of TGZ (200 mg/kg/day, 7 days) had no impact on the expression of monitored genes, except that Cyp7A1 was significantly increased.

Bsep KO had no effect on the evaluated serum chemistry parameters, as no changes were observed between WT and KO rats administered with the dosing vehicle (Table 3). When dosed with TGZ for 7 days, ALT was slightly increased in both WT and KO rats, but the changes were less than 1.5 fold in both cases. All other values, which included liver function indicators (AST, ALP and TBIL), as well as the kidney function markers (BUN and creatinine), were not significantly different between WT and KO rats, between TGZ and vehicle treatment, or between Day-1 and Day-7.

The plasma profiles of 13 bile acids were evaluated in WT and KO rats dosed with vehicle or TGZ on Day-1 and Day-7 (Supplemental Figure 3). The concentrations of liver bile acids were also quantified at the end of experiments. In animals treated with vehicle control, the majority of the
bile acids measured in the plasma displayed similar profiles between Day-1 and Day-7 in WT rats, except that DCA was slightly reduced, and TCDCA and T-αMCA were increased. Similarly, the bile acid profile of KO rats on Day-1 appeared to be the same as that on Day-7 (Figure 4A). The Bsep KO had complicated impacts on bile acid profiles as compared to WT rats. Plasma exposures of GCA, GDCA, GCDCA and TDCA were significantly decreased on both Day-1 and Day-7, while T-β-MCA was significantly increased by Bsep KO. TGZ administration had differential impacts on plasma bile acid levels between WT and KO rats. Following the first TGZ dose in WT rats, the plasma exposures of CA, DCA, βMCA, TβMCA were significantly higher than that in the rats with vehicle treatment (Figure 4B). Interestingly, repeated TGZ administration attenuated the changes of these bile acids as their concentrations restored to the levels comparable to the vehicle group. On the other hand, GCA, GDCA, GCDCA, TCDCA, TαMCA were significantly increased only in WT rats with repeated TGZ dose. In KO rats, a single dose of TGZ had minimal effects on the bile acid profiles, as compared to vehicle control in KO rats (Figure 4C). However, CA, TDCA, TCDCA, αMCA, TαMCA and TβMCA in plasma of KO rats were significantly altered by repeated doses of TGZ. In liver, the impact of Bsep KO on bile acid concentrations followed a similar movement to that observed in the plasma (Figure 4D). Following repeated TGZ treatment, liver concentrations of CA, DCA, CDCA and βMCA in WT rats had the trend to increase. In KO rats, only TβMCA was significantly increased with repeated TGZ doses.
Discussion

Bile acids are a class of steroid acids, which are primarily synthesized in liver and excreted into bile to aid the absorption of fats, lipids and similar nutrients in small intestine (Russell, 2003). The biliary secreted bile acids are further metabolized by gut microbiota and the majority of secreted bile acids are reabsorbed in the ileum and recycled back to the body. Bile acid synthesis is a major route for body cholesterol elimination. Meanwhile, bile acids are the driving force for bile flow, which is important for the elimination of metabolic wastes, such as bilirubin (Kullak-Ublick et al., 2000). Due to the physicochemical characteristics, bile acids possess low membrane permeability in general and require the assistance of transporters to cross cell membrane. Therefore, interruption of bile acid transport can cause severe clinical consequences, such as hyperlipidemia, cholestasis, cholecystitis and diarrhea, depending on the bile acid transport site of the interruption (Li and Chiang, 2012; Chiang, 2013). As BSEP is the key regulator secreting bile acids in liver, interference with BSEP activity is expected to influence the body bile acid profiles.

In the present studies, the Bsep KO rats was developed using Zinc Finger Nuclease technology to delete 8 nucleotides from the Bsep gene. This deletion is expected to generate a stop codon on exon 5 and to result in a premature termination of transcription and subsequently a functionally deficient Bsep protein. As confirmed by RT-PCR, no Bsep mRNA was detected in the liver of KO rats when applying PCR probes specifically targeting the deleted nucleotides, indicating the successful removal of the intended region. The full cDNA sequencing data further confirmed our gene expression results that 8 nucleotides were deleted in the Bsep gene in KO animals. Of interest, while sequencing the Bsep gene in both WT and KO rats, we identified a splice variant form missing 49 nucleotides at exon 3 (Supplementary Figure 2). Although mutation was not determined on ZFN targeting region, it is possible that the previously detected trace amount of
Bsep protein using LC/MS-MS quantitation method could be from an unidentified variant of reported Bsep gene (NCBI reference sequence: NM_031760), which was not disrupted in this KO model (Cheng et al., 2016b).

Bsep KO had a substantial impact on the expression of liver transporters in rats. A significantly higher amount of Bsep mRNA expression was observed in KO rats than in WT rats using the probes targeting outside of the ZFN modification region, representing a feedback response of reduced Bsep function in KO rats. The expression of Mrp2, Mrp3, Mrp4, P-gp, Bcrp and Ntcp genes was altered in the liver of KO rats. As a result, the pre-existing gene regulations of liver transporters impacted the disposition of orally administered TGZ in Bsep KO rats. TGZ is known to be metabolized to TS, TGZ glucuronide and TGZ quinone in rats and humans, of which TS is the predominant metabolite (Kawai et al., 1997; Loi et al., 1999). In this study, we observed no difference in the pharmacokinetic profiles of TGZ between WT and KO rats and between single and repeated doses, suggesting that transporter regulation detected in KO rats had no impact on TGZ disposition. However, plasma exposure of TS in KO rats was 7.6- and 9.3-fold lower than in WT rats on Day-1 and Day-7, respectively. Given the fact that plasma clearance rates of TGZ were comparable between WT and KO rats, the lower TS exposure in KO rats is not likely due to reduced metabolism, but from increased hepatic secretion by up-regulating hepatobiliary efflux transporters. This hypothesis was supported by the in vitro data that formations of TS were similar in liver S9 fractions prepared from WT and KO rats. Furthermore, the decrease in TS exposure was consistent with the increased Mrp2 expression in the liver of KO rats, as Mrp2 is the transporter for TS biliary excretion (Kostrubsky et al., 2001). Therefore, caution should be taken to apply KO animals in drug pharmacokinetic and toxicity evaluations since regulations of other
transporters and factors could occur to compensate the absence of interested transporter, which could complicate the disposition of tested drugs and then lead to misguided conclusions.

The impairment of Bsep function is expected to produce an accumulation of bile acids in hepatocytes, which will activate the nuclear receptor Fxr and then the downstream regulator Shp, leading to the upregulation of efflux transporter genes and downregulation of bile acid uptake and synthesis (Tu et al., 2000; Poupon, 2012). In this study, impairment of Bsep activity by Bsep KO and repeated TGZ treatment had sophisticated and differential regulations on liver transporters, enzymes and gene regulators that are associated with bile acid homeostasis, as illustrated in Figure 5. It should be noted that the evaluation of the gene expression was conducted at the end of experiments, 24 h after the last dose; therefore, the monitored gene expression reflects the overall changes including the compensatory effects following repeated doses. Nevertheless, the gene changes are generally agreed with previous reports, except for Cyp7A1 (Poupon, 2012).

Cyp7A1 is the rate limiting enzyme in bile acid synthesis from cholesterol and is suppressed by Shp, which is induced by Fxr activation (Goodwin et al., 2000; Rodrigues et al., 2014). Surprisingly, the expression of Cyp7A1 was significantly upregulated in WT and KO rats with repeated TGZ administered, although the level seemed to be slightly suppressed in KO rats compared to in WT rats, probably as a result of higher Shp in KO animals. The mechanism for this Cyp7A1 upregulation remains unknown. One of the possible explanations could be that repeated TGZ administration complicated the regulation of Cyp7A1 through the engagement of other pathways, i.e. the activation of the peroxisome proliferator activated receptors (PPAR). In fact, TGZ is a potent agonist of PPARγ developed for the treatment of diabetic and inflammatory disease (Escher and Wahli, 2000; Coyle and Kinsella, 2006). It was reported that activation of PPARγ could induce CYP7A1 expression to enhance cholesterol metabolism (Duan et al., 2012).
Further studies are necessary to understand the complicated mechanism of TGZ effects on Cyp7A1 expression. Since the bile acid pool in humans is more hydrophobic and toxic, the induced Cyp7A1 expression by TGZ may increase bile acid production and raise the liver load of bile acid accumulation and stress when a compensatory mechanism is not sufficient.

The changes of transporters, enzymes and regulators following Bsep impairment are correlated with the observed bile acid alterations. Following a single dose of TGZ, plasma exposures of CA, DCA, βMCA and TβMCA were significantly increased in WT rats. In contrast, the single dose of TGZ had no effect in Bsep KO rats. The differential impacts of single TGZ administration between KO and WT rats indicate that the increase of bile acids in WT rats could be due to Bsep inhibition by TGZ and/or TS. In fact, the average liver concentration of TS was 9.7 µM in WT rats at 24 h post the last dose, with a liver to plasma ratio of 7.7. Assuming that this ratio is consistent and the intracellular unbound fraction of TS is 0.0032, the predicted maximum liver free concentration of TS is 0.25 µM, slightly greater than the reported TS IC\textsubscript{50} value (0.2 µM) against rat Bsep (Funk et al., 2001a; Guo et al., 2017). Actually, the maximum liver TS concentration could be higher than this prediction, because the high concentration of TS at T\textsubscript{max} may exceed the excretion capacity of Mrp2. Interestingly, the altered plasma concentrations of bile acids by a single dose of TGZ was attenuated in WT rats following repeated TGZ doses. The increased plasma exposures of CA, DCA, β-MCA, T-β-MCA observed with a single dose of TGZ returned to the levels comparable to the control group, suggesting the acquired compensatory regulation of bile acid homeostasis followed by the long term inhibition of Bsep by TGZ/TS. Baat, which catalyzes the formation of glycine or taurine conjugated bile acids, remained unchanged in all treatments. Therefore, the observed changes in taurine and glycine conjugated bile acids in the plasma and liver of KO rats were likely due to altered transporter activity. Meanwhile, TGZ appeared to have no impact on
transporter expression, and then taurine and glycine conjugated bile acids were minimally altered in TGZ treated rats.

Interference of BSEP function is thought to be one of the mechanisms for TGZ induced liver injury in humans (Smith, 2003). A mechanism based systemic pharmacology model demonstrated that the serum ALT levels were found to be sensitive to the inhibition of BSEP with the dose of TGZ in humans (Yang et al., 2014). Here we found that the hydrophilic and less toxic bile acids were changed dramatically by Bsep KO and repeated TGZ treatments in rats, while the hydrophobic and cytotoxic bile acids, DCA and CDCA, were minimally affected (Schölmerich et al., 1984; Perez and Briz, 2009). This agrees with the serum chemistry data that no signal of liver toxicity was observed. Regulation of transporters and bile acid compositions seem to be the protective mechanisms for rats against Bsep impairment, and it may be one of the reasons why rat failed to serve as a good preclinical model to predict DILI in humans, especially in the aspect of BSEP inhibition. However, the observed individual bile acid profiles in plasma seem to correlate with Bsep impairment in rats, and therefore could be biomarkers to evaluate Bsep function in early drug discovery. It has been reported that the plasma levels of selected bile acids, such as GCA and GCDCA, are strongly associated with OATP1B activity in cynomolgus monkeys and humans (Xiang et al., 2009; Chu et al., 2015; Yang et al., 2016; Yee et al., 2016). Further investigations are required to identify the specific bile acids as biomarkers for BSEP function and to confirm whether the results in rats can be translated to humans.

In summary, the expression of liver transporters was significantly impacted by Bsep KO in rats, leading to the altered disposition profiles of TGZ sulfate in plasma and liver. Repeated TGZ treatment had minimal impact on liver transporters, but significantly upregulated Cyp7A1. These compensatory regulations were correlated with the changes of individual bile acids in plasma.
Additionally, this is the first report that TGZ can increase Cyp7A1 expression, probably through activation of the PPARγ nuclear receptor, which could be a potential explanation for TGZ-induced liver injury, in addition to the mechanism of BSEP inhibition in humans.
Conflict of interest

There is no conflict of interest to report.
Authorship Contributions

Participated in research design: Yaofeng Cheng, W. Griffith Humphreys, Jinping Gan, and Yurong Lai

Conducted experiments: Yaofeng Cheng, Shenjue Chen, Chris Freeden, Weiqi Chen, Yueping Zhang, and Pamela Abraham

Performed data analysis: Yaofeng Cheng, Jinping Gan, and Yurong Lai

Wrote or contributed to the writing of the manuscript: Yaofeng Cheng, David M. Nelson, W. Griffith Humphreys, Jinping Gan, and Yurong Lai
References


Figure Legends

**Figure 1**  Plasma concentrations of TGZ (A) and TS (B) on Day-1 and Day-7 in WT and KO rats following daily oral dose of TGZ (200 mg/kg/day) for 7 days (mean±SD, n=4-6).

**Figure 2**  Liver concentration and the in vitro formation of TS. A, liver concentrations of TGZ and TS on Day-7 in WT and KO rats following daily oral dose of TGZ (200 mg/kg/day) for 7 days; B, relative formation of TS (peak area ratio of TS over IS) in liver S9 prepared from WT and KO rats in the vehicle treatment group. Data are presented as mean±SD (n=5-6). **, p<0.01 when compared to WT rats.

**Figure 3**  Relative gene expression of liver transporters (A) and enzymes/nuclear receptors (B) in liver samples from WT and KO rats administered with daily oral dose of vehicle or TGZ (200 mg/kg/day) for 7 days. Data are presented as mean±SD (n=5-6). *, p<0.05; **p<0.01 and ***, p<0.001 when compared to WT rats in the same treatment group. #, p<0.05; ##, p<0.01 and ###, p<0.001 when compared to vehicle treatment in the same rat type. N.D., not detected.

**Figure 4**  Individual bile acid profiles in plasma and liver. A, plasma exposures (AUC₀₋₂₄) in WT and KO rats dosed with vehicle solution; B, plasma exposures (AUC₀₋₂₄) in WT rats administered with a single or 7 daily oral dose of TGZ (200 mg/kg/day); C, plasma exposures (AUC₀₋₂₄) in KO rats administered with a single or 7 daily oral dose of TGZ (200 mg/kg/day); D, liver concentrations on Day-7. Data are presented as mean±SD (n=4-6). *, p<0.05; **p<0.01 and ***, p<0.001 when compared to WT rats in the same treatment group. #, p<0.05; ##, p<0.01 and ###, p<0.001 when compared to vehicle treatment in the same rat type.

**Figure 5**  Proposed regulations of bile acid synthesis and transport in rat liver followed by Bsep KO (A) or TGZ treatment (B).
# Table 1 List of primers and probes for RT-PCR

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<th>Gene</th>
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<th>Accession number</th>
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Bsep knockoут probe

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Table 2 Pharmacokinetic parameters of TGZ and TS in WT and Bsep-KO rats on Day-1 and Day-7 following daily oral dose of TGZ (200 mg/kg/day, 2 mL/kg/day). mean±SD, n=4-6.

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<tr>
<th></th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (nM)</th>
<th>AUC&lt;sub&gt;(0-7)&lt;/sub&gt; (nM*h)</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
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<td><strong>TGZ</strong></td>
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<td>WT</td>
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<tr>
<td>Day-1</td>
<td>1.80±0.45</td>
<td>2080±343</td>
<td>15264±1686</td>
<td>9.00±7.96</td>
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<td>Day-7</td>
<td>1.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2605±364</td>
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<td>Day-1</td>
<td>1.50±1.22</td>
<td>2720±721</td>
<td>12293±3871</td>
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<td>Day-7</td>
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<td>2013±350</td>
<td>11508±6699</td>
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<td><strong>TS</strong></td>
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<tr>
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<tr>
<td>Day-1</td>
<td>2.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10186±2088</td>
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<tr>
<td>Day-7</td>
<td>1.00&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>76269±23501</td>
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<tr>
<td>Day-1</td>
<td>3.00±1.10</td>
<td>1335±470&lt;sup&gt;***&lt;/sup&gt;</td>
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<td>Day-7</td>
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<td>6338±2907&lt;sup&gt;***&lt;/sup&gt;</td>
<td>5.92±0.32&lt;sup&gt;##&lt;/sup&gt;</td>
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</table>

<sup>a</sup>, SD is not available as values are the same among individuals.

Statistical analysis were conducted with One-way ANOVA. *, p<0.05; **, p<0.01; and ***, p<0.001 when compared to WT rats in the same day. #, p<0.05; ##, p<0.01 when compared to Day-1 in the same rat type.
Table 3 Serum chemistry profiles in WT and Bsep-KO rats before administering (pre-dose) or after administering (post-dose) 7 consecutive daily doses of TGZ (200 mg/kg/day, 2 mL/kg/day) or vehicle control (2 mL/kg/day). mean±SD, n=4-5.

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<th></th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
<th>TBIL</th>
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<th>Creatinine</th>
<th>Cholesterol</th>
<th>Albumin</th>
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<tr>
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<td>U/L</td>
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<td>U/L</td>
<td>mg/dL</td>
<td>mg/dL</td>
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<tr>
<td>Vehicle Pre-dose</td>
<td>61.8±2.95</td>
<td>54.8±2.17</td>
<td>294±63.6</td>
<td>&lt;0.10a</td>
<td>12.4±0.55</td>
<td>0.23±0.01</td>
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<td>3.60±0.07</td>
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<td>Post-dose</td>
<td>77.4±7.13</td>
<td>64.6±5.98</td>
<td>312±60.8</td>
<td>&lt;0.10</td>
<td>16.0±1.41</td>
<td>0.27±0.00</td>
<td>36.6±2.07</td>
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<td>TGZ Pre-dose</td>
<td>66.8±2.28</td>
<td>49.4±7.09</td>
<td>260±25.8</td>
<td>&lt;0.10</td>
<td>16.6±1.82</td>
<td>0.28±0.01</td>
<td>36.4±6.52</td>
<td>3.64±0.18</td>
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<td>Post-dose</td>
<td>70.5±4.43</td>
<td>70.5±4.43</td>
<td>252±14.4</td>
<td>&lt;0.10</td>
<td>16.0±1.41</td>
<td>0.28±0.01</td>
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<tr>
<td>KO</td>
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<tr>
<td>Vehicle Pre-dose</td>
<td>62.8±5.22</td>
<td>45.4±5.37</td>
<td>299±43.6</td>
<td>&lt;0.10</td>
<td>13.6±1.67</td>
<td>0.25±0.00</td>
<td>92.8±8.87</td>
<td>3.68±0.11</td>
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<tr>
<td>Post-dose</td>
<td>74.8±9.50</td>
<td>49.4±5.41</td>
<td>273±52.9</td>
<td>&lt;0.10</td>
<td>15.0±0.71</td>
<td>0.27±0.01</td>
<td>87.6±11.2</td>
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<td>TGZ Pre-dose</td>
<td>64.0±6.04</td>
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<td>&lt;0.10</td>
<td>14.4±2.88</td>
<td>0.28±0.01</td>
<td>97.4±4.56</td>
<td>3.42±0.08</td>
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<td>Post-dose</td>
<td>70.0±8.46</td>
<td>65.4±11.19</td>
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<td>0.29±0.01</td>
<td>88.0±6.24</td>
<td>3.42±0.08</td>
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</table>

a, Values below the lower limit of quantitation (LLOQ= 0.10 mg/dL)
Figure 2

A

Liver Concentration (nM)

<LOQ

WT  KO  WT  KO

TGZ  TS

**

B

TS relative formation

WT
KO

Time (min)

0  10  20  30  40  50  60

0.0  0.5  1.0  1.5
Figure 3

A

B
Figure 4

A

B

C

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Figure 5